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Limited phylogeographic structure in a flightless, Appalachian chalcidoid wasp, *Dipara trilineata* (Yoshimoto) (Hymenoptera, Diparidae), with reassessment of the male of the species

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Abstract

Dipara trilineata (Diparidae) is a widespread eastern North American parasitoid with apterous females and winged males. Despite its seemingly limited dispersal capabilities, phylogeographic analysis over southern Appalachia reveals little structure, with only limited population level isolation. DNA barcoding surveys also definitively associate the male of the species, which had previously been misattributed, and a description of the correctly associated male is provided.

Keywords

aptery, megabarcoding, parasitoid, phylogeography

Introduction

Dipara Walker, 1833 is a member of the Diparidae, a globally distributed family of about 130 species of Chalcidoidea (Desjardins 2007), recently elevated to family status from a subfamily of Pteromalidae (Burks et al. 2022). Among North American Chalcidoidea, *Dipara* are unusual with females that are flightless and ant-like in morphology. There is little literature on *Dipara* biology. For several years, Diparinae were thought solely to

parasitize soil dwelling Curculionidae (Coleoptera) based on the first documented host record in 1988 (Bouček 1988). However, several species have been successfully reared from mantid egg cases and tsetse fly puparia (Desjardins 2007). The host range of Diparidae needs further research to gain a better picture of parasitoid-host relationships. There are presently three native species of the genus *Dipara* known from North America (Desjardins 2007), *D. canadensis* Hedqvist, 1969, *D. nigriceps* (Ashmead, 1904), and *D. trilineata* (Yoshimoto, 1977). The European *D. petiolata* Walker, 1833, has also apparently been introduced to the region (Garrido Torres and Nieves-Aldrey 1999; Wiśniowski and Jirak-Leszczyńska 2021), though we aren't aware of specific records.

Dipara trilineata is the most common species of *Dipara* in the eastern United States. Described from Kentucky, there are also published records from Missouri, North Carolina, Florida, Arkansas, Texas, Oklahoma, Tennessee, and the District of Columbia (several of these under the now synonymous *Trimicrops bilineatus* Yoshimoto 1977 (Yoshimoto 1977; Bouček 1993; Desjardins 2007), and online photographic records from Louisiana and Quebec (based on identifiable photographic vouchers on BugGuide.net). This is a remarkably broad range for a species whose females are wingless and flightless. Finding the species to be abundant in leaf litter samples from the higher elevations of the southern Appalachian mountains, which function as a series of sky islands for many inhabitants (Browne and Ferree 2007; Hedin et al. 2015; Caterino and Recuero 2023), it seemed likely that *D. trilineata* would exhibit considerable genetic structure, and potentially cryptic species over its range. Using mitochondrial, barcode-region sequences from numerous southern Appalachian populations, we examine this hypothesis here.

We also address a mistaken attribution of males to this species. We have associated three male specimens from multiple populations unambiguously with females of *Dipara trilineata* through DNA barcodes, and find them to differ significantly from males originally described by Yoshimoto (1977). We rectify this error, and provide a new description of male morphology.

Methods

New data for this paper include 69 *Dipara trilineata* COI sequences, generated as part of an 'all-arthropods' metabarcoding study on the fauna of leaf litter in the high Appalachians, plus a small selection of other Chalcidoidea outgroups for rooting. Specimens of *D. trilineata* were identified using keys in Yoshimoto (1977). Descriptions of all described Diparidae with flightless females occurring in North America were carefully compared to our specimens. Significant character conflicts are found for all but *D. trilineata* (and its well-justified synonym *D. bilineatus* (Yoshimoto)), and the type and other known localities for these names correspond closely to the species as we treat it here. In preliminary analyses we included selected 'Diparidae' specimens from the Barcoding of Life Database (BoLD). However, finding that none of these affected the monophyly or polarity of the *D. trilineata* topology, we conducted most analyses without these.

Sequenced specimens came from our own recent collections (sampling map shown in Fig. 1), where we sifted leaf litter at sites ranging in elevation from 1300–2000 m

(~4500–6600 ft). The highest elevation sites (> 1500 m) were dominated by a canopy of red spruce (*Picea rubens*) and Fraser fir (*Abies fraseri*), with a litter layer composed mainly of their shed foliage. Lower localities were associated with more typical southeastern deciduous forest, with litters of oak, maple, birch, and *Rhododendron*. Litter samples were Berlese extracted until dry, and all specimens were collected into and preserved in 100% ethanol until extraction.

Prior to extraction, each specimen was imaged (images available at https://www.flickr.com/search/?user_id=183480085%40N02&desc&text=Dipara&view_all=1). Abdomens were subsequently punctured for digestion, and moved to a 96-well plate. Tissues were digested with lysis buffer and proteinase K (Omega BioTek, Norcross, GA), the liquid fraction then removed to a new plate and extracted using Omega BioTek's MagBind HDQ Blood and Tissue kit, eluting with 150 µL elution buffer. Voucher specimens were retained, labelled, assigned unique identifiers, and deposited in the Clemson University Arthropod Collection.

The data set includes sequences produced by Illumina and Nanopore methods. In both cases, mini- (421 bp) barcodes were amplified from the mitochondrial COI gene using the primers BF2-BR2 (GCHCCHGAYATRGCHTTYCC & TCDGGRTGNCCRAARAAYCA; Elbrecht and Leese 2017), corresponding to the downstream two-thirds of the standard barcoding region. Each PCR reaction was tagged with a unique combination of 9 bp indexes (Meier et al. 2016). All PCRs were conducted in 12.5 µL volumes (5.6 µL water, 1.25 µL Taq buffer, 1.25 µL dNTP mix [2.5 mM each], 0.4 µL MgCl [50 mM], 1.5 µL each primer, 0.05 µL Platinum Taq polymerase, 1 µL DNA template, with a 95 °C initial denaturation for 5 minutes, followed by 35 cycles of 94 °C (30 sec), 50 °C (30 sec), 72 °C (30 sec), and a 5 minute 72 °C final extension on an Eppendorf Gradient Mastercycler.

PCR products were combined and purified using Omega Bio-Tek's Mag-Bind Total Pure NGS Kit, in a ratio of 0.7:1 (enriching for fragments >300 bp). Illumina adapters and sequencing primers were ligated to PCR products using New England BioLab's Blunt/TA Ligase Master Mix. Resulting libraries were purified using Mag-Bind Total Pure NGS, quantified using a Qubit fluorometer, and sequenced on an Illumina MiSeq using a v.3 2 × 300 paired-end kit. For Nanopore MinION sequencing, libraries were prepared using the ligation sequencing kit LSK-112 (Oxford Nanopore Technologies, Oxford, UK), and loaded onto a v10.4 flowcell.

Illumina reads were processed with bbtools software package (https://jgi.doe.gov/data-and-tools/bbtools/; v38.87; Bushnell et al. 2017), trimming adapters, removing PhiX control reads, merging paired-end reads, filtering reads for the correct size, removing reads with quality score < 30, clustering sequences by similarity allowing 5 mismatches (~1%), and generating a final matrix in FASTA format. Nanopore reads were basecalled using the 'super-accurate' algorithm of Guppy (v6.1.2) running on Clemson's Palmetto cluster, then demultiplexed using ONTbarcoder v0.1.9 (Srivathsan et al. 2021), with minimum coverage set at 5. FASTA files from all sequencing runs were combined and aligned with the online version of Mafft v7 (Katoh et al. 2017) using the auto strategy. All barcode sequences have been deposited in GenBank, with accession #s listed in Suppl. material 1.

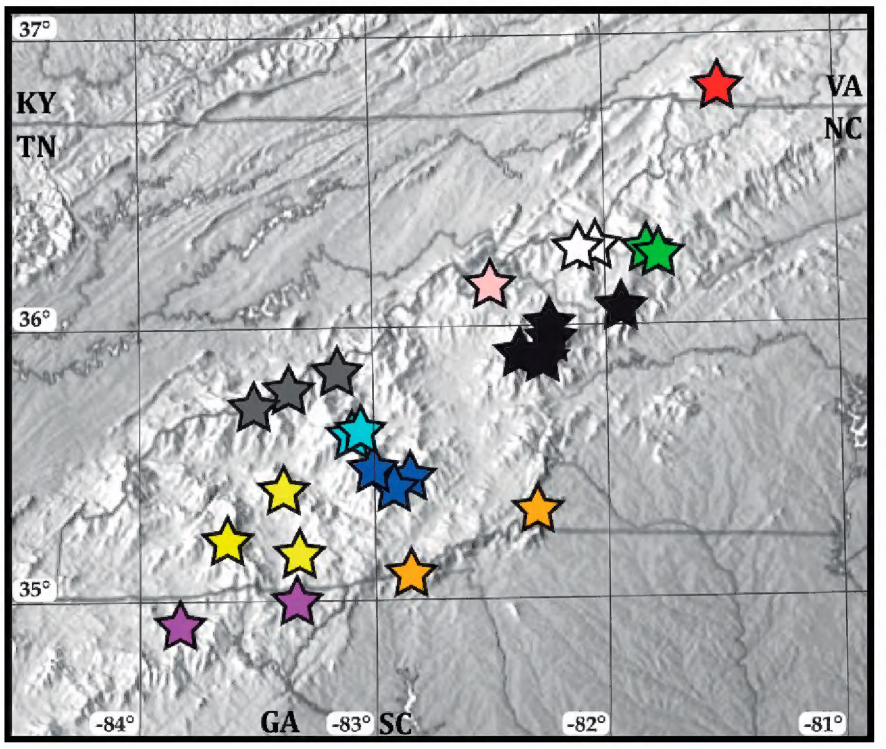

Figure 1. Map of all localities represented by COI sequences in the present study. Colors refer to those in trees in Figs 2, 3.

We produced a phylogeny using W-IQ-Tree (Nguyen et al. 2015; Trifinopoulos et al. 2016) under maximum likelihood criteria, applying a GTR+gamma model, with empirical base frequencies. Branch support was estimated using 10000 replicates of ultrafast bootstrapping (Minh et al. 2013). To assess relationships among haplotypes under a population genetic framework, a TCS haplotype network (Clement et al. 2000) was constructed using Popart (Leigh and Bryant 2015).

Results

Phylogenetic analyses that included a broader selection of Diparidae (not shown) from BOLD invariably resolved southern Appalachian *D. trilineata* as monophyletic, with no other available sequences very closely related. Sequences unidentified beyond 'Diparidae' from Thailand and Western Australia appeared more closely related to *D. trilineata* than did sequences of the Palaeartic *Dipara petiolata* or what appears (from a voucher photo in the BOLD database) to represent *D. canadensis* (from Virginia, USA).

Within *D. trilineata*, 69 individuals resolved into 35 distinct haplotypes. Divergences among them were remarkably low, with most less than 2% (uncorrected). The largest divergences were between a single individual from Brasstown Bald, Georgia (BBld.A.048) and most other sequences, at 4–6%. Comparisons to a couple other more divergent and well supported lineages (those from the Black Mts. in North Carolina and those from Whitetop Mt. in southwestern Virginia) were intermediate, ranging from 2–3.6%. Phylogenetic resolution was low and mostly weakly resolved (see Fig. 2). The deeply divergent individual from Brasstown Bald in northeastern Georgia was resolved as the sister to all other populations, although it differs in no obvious morphological characteristics. Among the latter, a single individual from a lower elevation locality in south-central North Carolina (Green

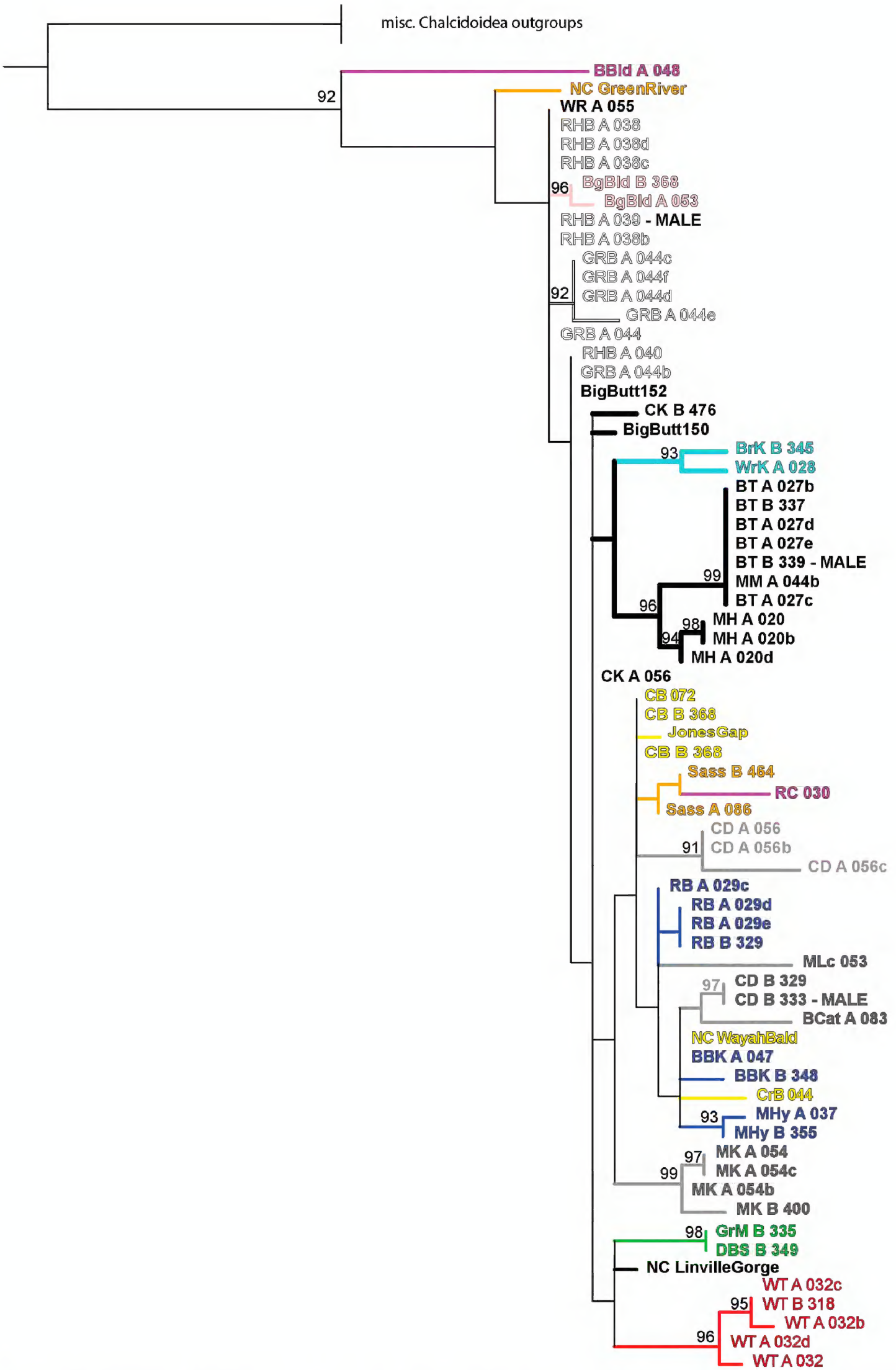

Figure 2. Maximum likelihood phylogeny of *Dipara trilineata* individuals, with locality abbreviations as in Suppl. material 1, and colors of OTUs keyed to localities shown in Fig. 1.

River) was sister to the remainder. More northern populations (Roan Highlands, Big Bald, Black Mountains, Grandfather Mt., and Whitetop) were broadly paraphyletic with respect to populations southwest of the Asheville Depression. Populations in the latter region were resolved into a few moderately to weakly supported lineages (mostly from single localities: Mt. Kephart, Mt. Hardy), but relationships among most are unresolved.

The haplotype network (Fig. 3) uncovers little population level structuring. Although only a couple haplotypes are shared across populations (Black Mts. and Roan Highlands by one haplotype, Nantahala Mts. and Great Balsam Mts. by another), few populations form tight clusters, and haplotypes from some widely separated localities (e.g., Celo Knob in the northern Black Mts. and Rabun Cliffs in north Georgia) are quite closely related (differing in that case by only two mutations).

Three male specimens (fully winged, with long filiform antennae), representing three different populations, were resolved as identical to one or more females from their respective populations, and can be considered definitively associated. These specimens conflict in several characters with the descriptions and figures presented in Yoshimoto (1977), then described as the males of the now synonymous *Trimicrops bilineatus*. The clearest point of contrast is in the antennal flagellum, shown in fig. 25 of Yoshimoto (1977: p.1053) as moderately elongate, with evenly cylindrical flagellomeres with surfaces covered with short setae, described as "filiform, densely pubescent with a single short annellus". In the specimens we attribute to *D. trilineata* (Fig. 4C–F) the antennae are

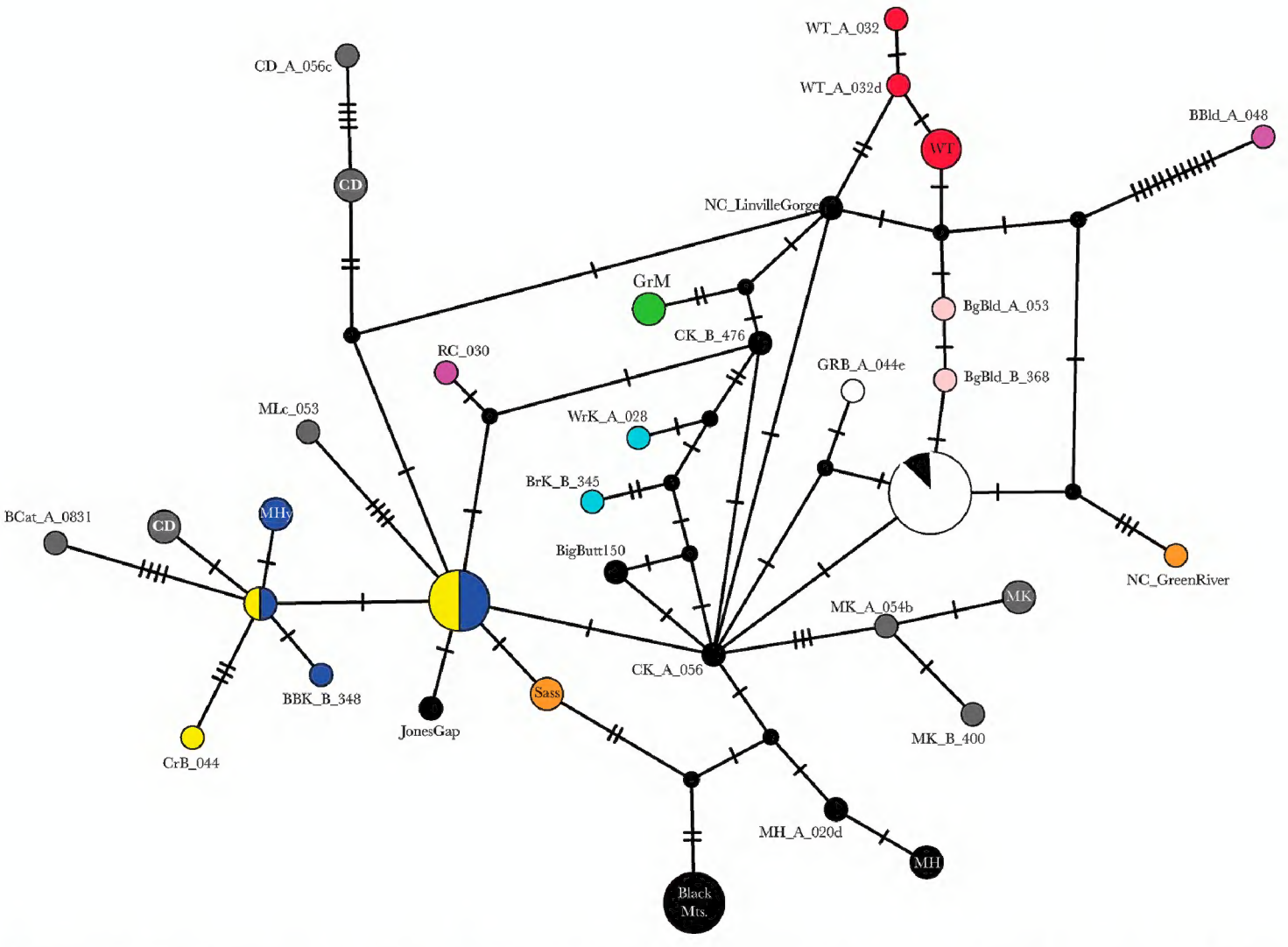

Figure 3. Haplotype network from TCS analysis, with sizes of circles proportional to number of individuals with that haplotype, and colors of circles keyed to localities as shown in Fig. 1.

much more slender, every flagellomere tapered basally and distally, and verticillate, with few very long setae borne in whorls (Fig. 4E). Yoshimoto also figures the wings in his Figure 8 (1977: p. 1050), showing the hind wing to be broadly rounded apically, while our *D. trilineata* has much narrower, apically subacute hind wings (Fig. 4F). Those are the only characters illustrated by Yoshimoto, but it is apparent that the described "allotype" male from the type locality represents a distinct species. The male he attributed to *Dipara pedunculata* (with antennae figured in Yoshimoto's Fig. 27; 1977: p. 1053), now considered a synonym of *D. canadensis*, matches our *D. trilineata* males much better than it does *D. canadensis* (the male antenna of which is shown in his fig. 26: (1977: p. 1053). Heydon and Bouček (1992), when synonymizing *D. pedunculata* with *D. canadensis*, previously noted some inconsistencies between Yoshimoto's (1977) description and female holotype. We suggest that the male presumed to represent Yoshimoto's *D. pedunculata* was a misidentified *D. trilineata*. *Dipara pedunculata* was described from Kentucky, well within the range of *D. trilineata*, so the two valid species must be sympatric there, and the original series of *D. pedunculata* a mix of *D. canadensis* and *D. trilineata*.

Comparing our confirmed males of *D. trilineata* directly to Yoshimoto's (1977) description of *D. pedunculata*, we note several other points of difference, and provide a brief re-description here (with slightly updated terminology).

Male (Fig. 4C–F): Head, mesosoma, and metasoma fuscous; legs (except mesocoxa), petiole, and bases of antennae yellowish, the antennae gradually darker from 3rd flagellar segment distad, mesocoxa also darker toward base; head almost hemispherical, very shallowly depressed above toruli, smooth and shining above, finely transversely reticulate below toruli, with scattered setae throughout; eyes prominent, eye height slightly more than half lateral head height, coarsely faceted; ocellar triangle wide, individual ocelli oval; clypeus outlined by disconnected series of punctures, convex, apical margin evenly rounded; mandibles tridentate; antennae inserted in front of middle of eye, slightly above middle of frons, toruli approximately equally separated from each other as from inner edge of eye; scape cylindrical, slightly curved, almost as long as pedicel and flagellomeres 2 and 3 combined; pedicel short, expanded to slightly wider than scape at apex, flagellomeres narrow basally and apically ('pedunculate'), but bulbous in basal half, tapered apically, with few (~6) long setae (about 1.5 times as long as flagellomere) inserted in an uneven series around bulbous base; entire antenna nearly as long as rest of body; neck transversely reticulate, bounded posteriorly by evenly curved, weakly impressed collar; notauli subcrenulately impressed, curving to meet along finely and deeply impressed mesoscutum-scutellar suture, the mesoscutum polygonally microsculptured between; frenal groove of scutellum only weakly indicated, but frenum smoother than polygonally microsculptured scutellum; propodeum with coarsely raised reticulate microsculpture; anterior insertion of petiole slightly narrower than posterior insertion, petiole about 3 times as long as maximum width, with weak longitudinal carinae; 1st gastral segment nearly half entire gastral length, 2nd–5th gastral segments subequal in length; forewing widening only slightly beneath costal cell, widening more abruptly beyond, anterior margin bent slightly forward at this point; submarginal vein bearing two conspicuous dorsal setae; marginal vein more densely setose, the setae directed distad at about 45°, their maximum length about ¼ maximum wing width; postmarginal vein

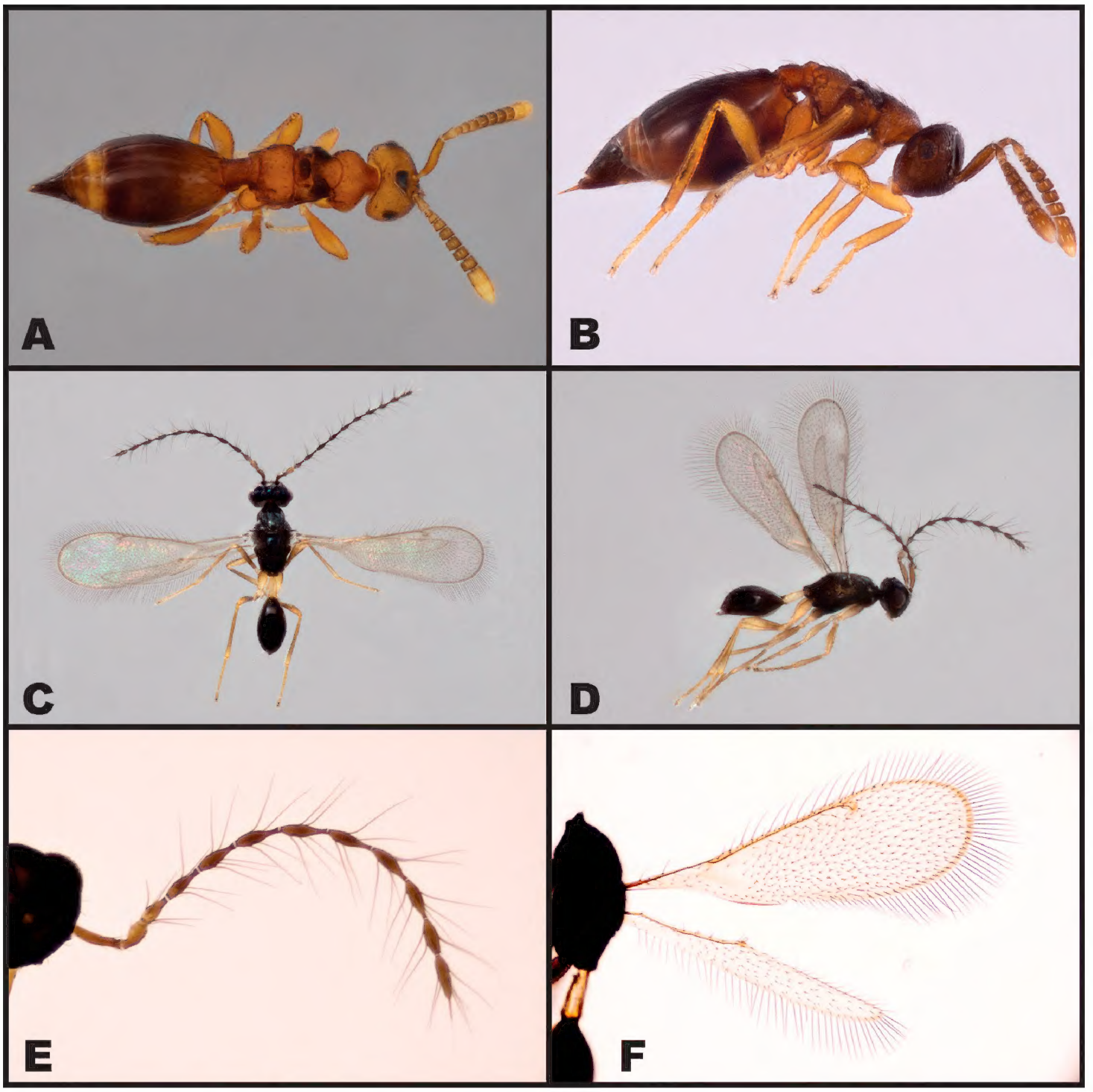

Figure 4. Female (**A, B**) and male (**C–F**) *Dipara trilineata* (Yoshimoto).

weak, fading evenly beyond short stigmal vein, stigma slightly expanded, uncus poorly developed; wing with sublinear series of short setae in basal cell, bare briefly within specular area, densely and evenly setose beyond; setae of apical and posteroapical margins of wing long, nearly half maximum wing width; hind wing about three-fourths length of forewing, posterior margin rounded, widened slightly beyond midpoint, narrowed to subacute apex, setae along posterior margin longer than width of hindwing membrane.

Material examined (males): North Carolina, Yancey County, Mt. Mitchell State Park, Big Tom near summit (35.7799, -82.2596), 7-Sep-2021 (CUAC000135520); North Carolina, Swain County, Great Smoky Mountains National Park, Clingmans Dome (35.5589, -83.4983), 14-Sep-2021 (CUAC000157203); North Carolina, Mitchell County, Roan High Bluff (36.0931, -82.1453), 15-Aug-2018 (CUAC000002974).

Other taxonomic remarks: No recent authors have addressed the mismatch in gender of *Dipara trilineatus* (sic). Walker's (1833) genus name would be feminine, appearing to be based on a Greek adverb used as a singular noun (S. Chatzimanolis, pers. comm.),

and virtually all usage from Walker's onward has used feminine species names. It is unfortunate that when synonymizing *Trimicrops* Keiffer with *Dipara* Walker, Desjardins (2007) did not properly amend 'trilineatus' to the singular feminine ending, but we do that here.

Discussion

Dipara trilineata is a remarkably widespread species for one having such seemingly limited dispersal capabilities. Our collections, along with reliable records from other sources reveal the species to cover much of the eastern US, extending from central Texas into southeastern Canada. As to state records, the species was previously unreported for Mississippi, Indiana, Georgia, South Carolina, Virginia, and West Virginia (Fig. 5).

Even more surprising is the relatively limited degree of population structuring, at least over the range we sampled. Some geographic clustering is evident, and a number of populations exhibit haplotype monophyly, but the overall patterns exhibit only loose correspondence with geography. One potential confounding factor is the relatively high haplotype diversity, as would be expected for a species with large population sizes. This could slow coalescence and limit phylogenetic resolution even if populations are largely isolated. But based on available data, there are no indications that *D. trilineata* represents a cryptic species complex, despite its flightlessness. If additional individuals from the more divergent lineages (BBld.A.048 or NC_GreenRiver) showed comparable genetic difference, more systematic morphological comparisons may reveal subtle differences not yet apparent.

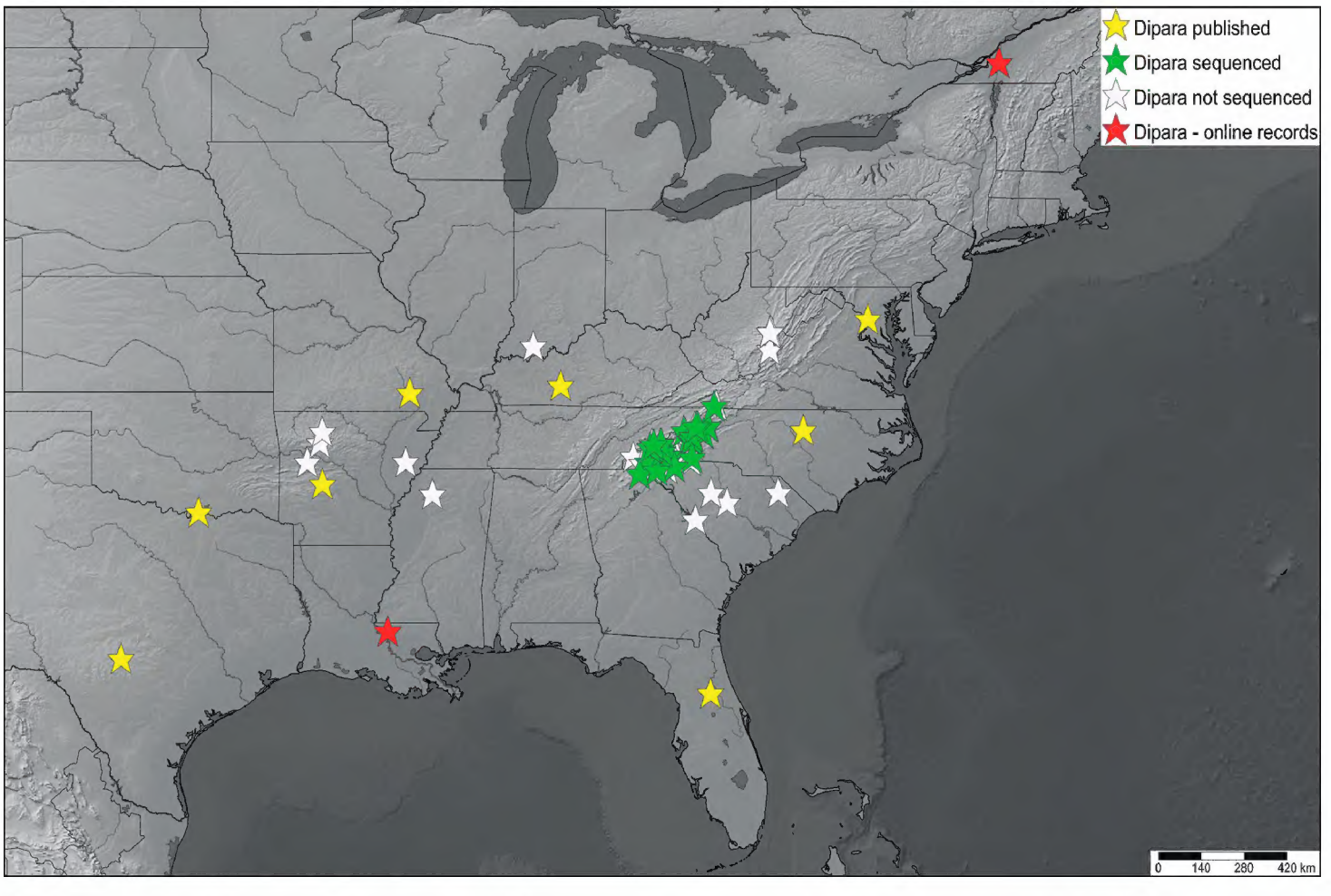

Figure 5. Total known distribution of *Dipara trilineata*, based on a combination of published, online, and newly contributed records.

The lack of phylogeographic structuring may provide some indirect hints as to host breadth. Even though individual *Dipara* females may not themselves be capable of long-distance dispersal, it is worth suggesting the potential for dispersal in the larval stage by a more mobile, flying or ballooning host, which would serve to reduce effective isolation (as has been shown for Dryinidae parasitoids of leafhoppers; Mita et al. 2012). Host records for *Dipara* to date include only non-mobile stages, eggs, larvae, and pupae (Desjardins 2007). But these already cover a considerable range, and more mobile hosts should not be ruled out.

As to potential host identities for *Dipara trilineata*, its general abundance over a wide range argues against any close host specificity. There are few other arthropod species in eastern US leaf litter that have so wide a distribution, occurring in such a wide range of microhabitats, although perhaps some of the spider species do (Recuero et al. 2023). Previous suggestions of weevil associations would not seem likely, at least not as a primary host, as weevils are poorly represented in our highest elevation samples. There are intriguing possibilities to better understand host/parasitoid relationships through metabarcoding approaches, such as detecting the DNA of a parasitoid as co-amplifying with that of its host (Miller et al. 2021), and the *Dipara* system would be a promising one to explore such potential.

Acknowledgments

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Supplementary material 1

Excel spreadsheet with specimen level data for all records of *Dipara trilineata* reported here

Authors: Michael S. Caterino, Nathan C. Arey

Data type: xlsx

Explanation note: Fields include source of record, project morphospecies code (searchable on Flickr), sex, Caterino lab DNA extraction number, GenBank accession number (where sequenced successfully), verbal locality description, decimal latitude/longitude, date collected, and unique CUAC voucher code.

Link: https://doi.org/10.3897/jhr.96.115001.suppl1